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Onset of *C. elegans* Gastrulation Is Blocked by Inhibition of Embryonic Transcription with an RNA Polymerase Antisense RNA

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Cleavage and gastrulation initiation in *Caenorhabditis elegans* embryos are characterized by an invariant temporal and spatial pattern of cell divisions and cell movements. Although bulk embryonic transcription does not begin until gastrulation onset, some transcription can be detected as early as the 4-cell stage. To determine whether any early transcripts are required for normal cleavage-stage patterning, we blocked transcription in embryos by injecting hermaphrodite parental gonads with RNA antisense to the *ama-1* gene, which encodes the large subunit of RNA polymerase II. This treatment prevented the expression of a reporter gene driven by an early embryonic promoter but did not detectably perturb the maternally controlled segregation of the germ line P granules or the pattern of cell division through the first four cleavages. In the fifth cell cycle, however, the two endodermal precursor (E) cells divided early and abnormally and failed to initiate gastrulation. The embryos arrested between the sixth and seventh cell cycles with less than 100 cells. These results indicate that embryonically transcribed gene products are required for gastrulation initiation. They also demonstrate the efficacy of a method for blocking embryonic transcription that may be useful in other organisms.

INTRODUCTION

Embryogenesis is directed by the asymmetric localization of maternally contributed gene products and the subsequent cell-specific activation of embryonically transcribed regulators (reviewed in Davidson, 1990). The transition from maternal to embryonic control is not necessarily defined by the onset of bulk transcription. For example, in *Drosophila*, high rates of transcription commence at cycle 14, just prior to the onset of gastrulation (Edgar and Schubiger, 1986). However, several embryonically transcribed genes are highly expressed before this time and have been shown to function at the cellular blastoderm stage (Weir and Kornberg, 1985; Knipple *et al.*, 1985; Ingham *et al.*, 1985; Jäckle *et al.*, 1986; Howard and Ingham, 1986; Harding and Levine, 1988). In the leech *Helobdella*, embryonic transcription is required to specify spindle orientation of the teloblast progenitor D′ at stage 5, several divisions before the global activation of embryonic transcription at stage 7. However, once the teloblasts have been generated, they can divide asymmetrically to produce blast cells in the presence of transcription inhibitors, suggesting that the time between the first requirement for embryonic transcription and the last cellular event under maternal control spans several divisions (Bissen and Weisblat, 1991; Bissen and Smith, 1996).

In embryos of the nematode *Caenorhabditis elegans*, the first requirement for embryonic transcription has not been established. In these embryos, the orientation and timing of the early blastomere divisions are invariant, and the embryonic lineages of all cells in the hatching first-stage larva have been completely defined (Deppe *et al.*, 1978; Sulston *et al.*, 1983). By the 24-cell stage, the five major somatic lineages (AB, MS, E, C, and D) and a unique germ line cell (P_4) have been established. At the 26-cell stage, approximately 1.5 hr postfertilization, gastrulation initiates when the two gut precursors Ea and Ep begin to move from the ventral surface into the blastocoel (cells are named according to ancestry; Ea and Ep are the anterior and posterior daughters, respectively, of the gut lineage founder cell E). Over the next 4 hr, as cell division continues, the mesodermal (MS, C, and D descendants) and germ line (P_4) precursors ingress through a ventral cleft to form the three germ layers of the embryo (Sulston *et al.*, 1983). Organogenesis

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and morphogenesis initiate during this period and proceed for the next 8 hr, as the forming worm elongates and secretes a cuticle (for review, see Wood, 1988).

Analysis of maternal-effect embryonic lethal mutations has identified two partially overlapping classes of genes that control early patterning during the cleavage stage: those responsible for asymmetric partitioning of cytoplasmic components (e.g., the *par* genes; Kemphues *et al.*, 1988; Etemad-Moghadam *et al.*, 1995; Guo and Kemphues, 1995) and those that more directly specify early cell fates (e.g., the *skn-1* and *pie-1* genes; Bowerman *et al.*, 1992; Mello *et al.*, 1992; 1994; Lin *et al.*, 1995). Embryonic transcription of at least one gene has been demonstrated at the 4-cell stage (Seydoux and Fire, 1994), and RNA synthesis can first be detected by radioactive UTP incorporation at the 8- to 12-cell stage (Edgar *et al.*, 1994). Analysis of run-on transcription in preparations of nuclei from early embryos suggested that 20–30 very early transcript (*vet*) genes are active prior to gastrulation onset (Schauer and Wood, 1990), and more recent experiments using *in situ* hybridization and reporter constructs (Seydoux *et al.*, 1996; J. Pettitt and W.B.W., unpublished results) show that several of these transcripts are being made in all somatic cells by the 26-cell stage.

Nevertheless, no cellular event prior to the 100-cell stage has been clearly shown to be dependent upon embryonic transcription. Analysis of embryos homozygous for chromosomal deficiencies representing approximately half the genome did not identify any genomic regions required for specifying either the timing or orientation of early cleavages (Storfer-Glazer and Wood, 1994). Furthermore, inhibition of RNA polymerase II with α-amanitin did not significantly alter the timing of early blastomere divisions in devitellinized, cultured embryos, which arrested cell division at approximately 100 cells in the presence of the drug (Edgar *et al.*, 1994). These results suggested that while *C. elegans* embryos are transcribing many genes by the 26-cell stage, maternal gene products might be sufficient to dictate at least the mechanics of the first several cell cycles and the onset of gastrulation. However, α-amanitin experiments have the important limitation that the eggshell and vitelline membrane must be removed to expose the embryo to the drug. Disruption of the vitelline membrane, while not significantly affecting the timing and asymmetries of early cleavages, does perturb normal cell contacts (Edgar and McGhee, 1988; Edgar, 1995) and prevents initiation of gastrulation (Schierenberg and Junkersdorf, 1992).

To examine further the requirement for embryonic transcription in the specification of early cleavage patterns and cell movements, we sought a molecular or genetic approach that would specifically inhibit RNA polymerase in the intact embryo. The *ama-1* gene, which encodes the large subunit of RNA polymerase II, has been mutationally identified (Rogalski and Riddle, 1988; Rogalski *et al.*, 1988) and cloned (Bird and Riddle, 1989). However, no mutations have been found that allow elimination of *ama-1* function in early embryos without impairment of oogenesis as well. Therefore, we attempted to employ an antisense strategy for eliminating embryonic RNA polymerase II. Antisense RNAs have previously been used successfully in *C. elegans* to decrease specific expression of both maternally and embryonically expressed genes and thereby mimic loss-of-function mutations (e.g., Fire *et al.*, 1991; Guo and Kemphues, 1995; Lin *et al.*, 1995).

In this study, we injected antisense *ama-1* RNA into the gonads of wild-type hermaphrodites to block maternal and early embryonic translation of mRNA for the RNA polymerase II large subunit. The resulting *ama-1* antisense embryos do not express an embryonically transcribed reporter gene and arrest at a stage similar to that of cultured embryos treated with α-amanitin, indicating that the antisense RNA effectively blocks embryonic function of RNA polymerase II. Observation of these embryos prior to their arrest reveals that the earliest partitioning events and cleavage patterns are not affected, but that the E (gut) lineage exhibits failure of normal cell-cycle regulation, spindle orientation, and gastrulation initiation.

MATERIALS AND METHODS

C. elegans Strains and Culture

C. elegans strains were maintained according to Sulston and Hodgkin (1988). Those used were the wild type Bristol (N2) strain and a transgenic derivative, BW1640, which carries an integrated *vet-6::lac Z* reporter construct (J. Pettitt and W.B.W., unpublished results).

DNA Constructs and in Vitro Transcription

The *ama-1* cDNA subclones DB19 and DB17 were generously provided to us by David Bird. pJ101 was constructed by ligating the 2-kb *Eco*RI fragment from DB19 (see Fig. 1) into T7/T3 α18. pJ102 was constructed by cutting DB17 with *Bsr*GI and *Sma*I to excise part of the 3′ UTR and the poly(A) tail and religating the pGem7Zf-based vector. pAS07 includes the entire coding region of the *C. elegans her-1* gene, 8 bp of the 3′ UTR, and 38 bp of the 5′ UTR (A. Streit and W.B.W., unpublished results).

ama-1 and *her-1* constructs were linearized at one end of the cDNA by restriction enzyme digestion. *In vitro* transcription of RNA was performed by mixing 1 μg linearized DNA template with 2 μl 5 m*M* dNTPs, 2 μl (10 μg/μl) BSA, 2 μl 100 m*M* DTT, 4 μl 5× transcription buffer (Promega), 0.5 μl (40 U/μl) RNAsin (Promega), 1 μl $m^7G(5')ppp(5')$ G (0.5 A_{250} U/μl; Boehringer-Mannheim), and either 0.5 μl SP6 RNA polymerase or 0.5 μl T7 RNA polymerase (each 15 U/μl; Boehringer-Mannheim). After incubation at 37°C for 1 hr, another 0.5 μl RNA polymerase was added, and incubation was continued for a second hour. The DNA template was digested by addition of RNase-free DNaseI (Boehringer-Mannheim) for 15 min at 37°C. RNA was extracted with acid phenol and chloroform, precipitated with 0.8 *M* LiCl and 2.5 vol of 100% EtOH and resuspended in diethylpyrocarbonate-treated water. The final concentration of RNA was determined with a spectrophotometer.

RNA Injections and Scoring of Progeny

Young adult hermaphrodites were selected for injection. Approximately 50 pl of an RNA solution was injected into each arm of each hermaphrodite gonad, and the worms were allowed to recover for 12–16 hr at 20°C before any of their progeny embryos were scored. Animals that died or sustained obvious damage to the gonad were not included in the analysis. The injected hermaphrodites were transferred singly to new plates every 12 hr for the duration of the scoring period (see legends for Tables 1 and 2). Eggs laid on these plates were counted immediately after the hermaphrodite was transferred, and the phenotypes were scored 20 hr later under the dissecting microscope. Embryos that arrested with fewer than 300 cells and without visible signs of differentiation or morphogenesis were defined as early arrest. Embryos that underwent some morphogenesis but died were defined as late embryonic lethal. Embryos that hatched into normally formed L1 larvae were defined as viable.

Immunofluorescence Microscopy

All procedures were carried out at room temperature, unless otherwise specified. Gravid hermaphrodites (N2 or BW1640) were transferred into a drop of M9 on a gelatin subbed slide and cut with a scalpel to release the embryos. Embryos were overlaid with an 18 × 18 mm glass coverslip and dipped in liquid nitrogen for 10 sec. The coverslips were popped off with a scalpel blade, and the slide was then fixed for 20 min in ice-cold methanol and air dried for 15 min. The samples were blocked in 50% normal goat serum in PBS with 0.5% Triton X-100 (TPBS) for 30 min. The anti-P granule antibody K76 (Strome and Wood, 1983) was placed on the slides undiluted, and samples were incubated overnight at 4°C. Slides were washed three times for 10 min each in TPBS. Samples were incubated for 45 min with donkey anti-mouse IgG secondary antibody (Jackson Immunoresearch), conjugated to rhodamine, and diluted 1:200 in TPBS. Slides were then washed three times for 10 min each in PBS, incubated for 5 min in 1 μg/ml diamidinophenylindole (DAPI) in PBS, and rinsed briefly in PBS. Samples were mounted under glass coverslips in Gelvatol (Monsanto) with 2% *n*-propyl galate to minimize bleaching. Stained specimens were viewed with a Leica DX-4 microscope equipped for flourescence microscopy.

Staining for β-Galactosidase Activity

BW1640 embryos were either transferred by mouth pipette from cut hermaphrodites to gelatin subbed slides and fixed as above or samples were used that had already been fixed and processed for K76 staining. Samples were stained with 100 μl of 0.2 *M* sodium phosphate buffer, pH 7.5, containing 10 m*M* $MgCl_2$, 5 m*M* $K_4Fe(CN)_6$, 5 m*M* $K_3Fe(CN)_6$, 0.04% SDS, and 0.24% X-gal (Fire *et al.*, 1990) at room temperature for 6–12 hr and then rinsed in 1 μg/ml DAPI in PBS prior to mounting under Gelvatol as above. Nuclei were counted by DAPI staining to stage embryos.

Lineage Analysis by 4D Microscopy

The development of embryos was recorded using a multi-focal-plane time-lapse video microscopy system (4D microscope), consisting of a Leica DX-4 microscope equipped with Nomarski optics, a Kohu video camera, and a computer controlled focusing system as described by Fire (1994). Embryos were mounted on a 4% bactoagar pad on a glass slide, overlaid with an 18 × 18 mm glass coverslip, and sealed with silicone grease (Sulston and Hodgkin, 1988). A series of through-focus optical sections in steps of 1 μm was recorded every 30 sec for 3–5 hr, while maintaining the embryo at 24.5 ± 1°C.

RESULTS

ama-1 Antisense RNAs Inhibit Embryonic Transcription and Cause Early Embryonic Arrest

To determine whether embryonic transcription would be inhibited by *ama-1* antisense RNA, we reverse-transcribed the DB19 *ama-1* cDNA and injected the RNA into both arms of the gonads of young adult hermaphrodites. The DB19 construct includes most of the *ama-1* coding sequence but does not include the transpliced leader sequence SL1 (Krause and Hirsh, 1987) or the 3′ untranslated region (Fig. 1). After 12 to 16 hr we transferred each injected hermaphrodite to a fresh plate and analyzed the phenotypes of the subsequently produced self-progeny embryos. Injection of a 5 ng/μl solution of antisense RNA caused 96% of the self-progeny embryos to arrest between the sixth and seventh cell cycle (90–100 cells) with no visible signs of cellular differentiation. This phenotype is termed ''early arrest'' in Table 1. Injection did not significantly decrease the number of eggs laid per hermaphrodite compared to uninjected controls (data not shown), and the phenotypes were consistent from the time we began scoring the embryos until the hermaphrodites ceased to lay eggs.

The similarity between the cell-cycle arrest phenotypes of *ama-1* antisense embryos and devitellinized embryos cultured in the presence of α-amanitin (Edgar *et al.*, 1994) suggested that the injected RNA was inhibiting embryonic transcription. To test this possibility more rigorously, we injected DB19 *ama-1* antisense RNA into hermaphrodites carrying an integrated *vet-6::lacZ* construct, which is strongly expressed in somatic blastomeres by the 28-cell stage (Seydoux *et al.*, 1996; J. Pettitt and W.B.W., unpublished results). When control embryos from uninjected hermaphrodites were stained for *vet-6::lacZ* expression, β-galactosidase was detectable in all but 5% of the embryos between the 28- and 100-cell stages (n = 130; Fig. 2A). In contrast, 96% of the *ama-1* antisense embryos did not detectably express the reporter gene (n = 186; Fig. 2B). Younger embryos (28- to 50-cell) were not overrepresented among the 4% that did stain, which we presume to be embryos that would not have arrested (also 4%; see previous paragraph). These results indicate that expression of the reporter is strongly inhibited by injection of *ama-1* antisense RNA, from the 28-cell stage onward.

To assess the specificity of this effect, we performed three sets of control experiments. First, to address the possibility that some of the embryonic lethality might result from physical injury to the maternal parent or from nonspecific effects of RNA injection, we injected hermaphrodites with

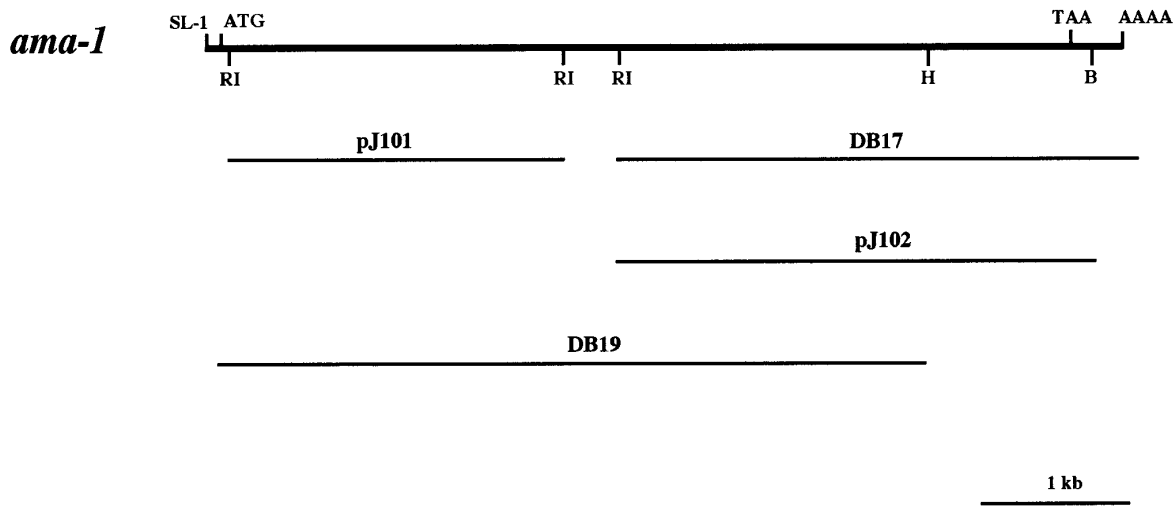


FIG. 1. Map of injected *ama-1* antisense RNAs. The positions of the splice leader sequence (SL1), the translation initiation methionine (ATG), translation stop codon (TAA), and the poly(A)tail (AAAA) in the sense *ama-1* mRNA are indicated, as well as the *Eco*RI (RI), *Hin*dIII (H), and *Bsg*I (B) restriction sites in the *ama-1* cDNA. The DB19 cDNA includes 40 bp of 5′ untranslated sequence, but not the SL1 splice leader sequence. The DB17 cDNA includes the entire 3′ untranslated sequence and 18 bp corresponding to the poly(A) tail. The pJ102 cDNA includes only part of the 3′ untranslated sequence and none of the poly(A) tail sequence.

340 ng/μl of RNA antisense to *her-1*, an embryonically transcribed sex-determining gene that is not essential for viability (Perry *et al.*, 1993). Of the progeny embryos examined, only 2% failed to hatch, and only 0.3% arrested with fewer than 300 cells (Table 1), arguing against nonspecific effects of RNA injection.

As a further control for specificity, we carried out similar injections with DB19 *ama-1* sense RNA. For reasons that are still unclear, sense RNA injections in *C. elegans* often result in loss-of-function phenotypes similar to those caused by the corresponding antisense RNA (Fire *et al.*, 1991; see Discussion). In our experiments, sense RNA also caused some embryonic arrest. However, while injection of a 2 ng/μl solution of antisense RNA resulted in 82% lethality, injection of sense RNA at the same concentration resulted in only 7% lethality of progeny embryos. Injection of a 5 ng/μl solution of sense RNA caused 23% lethality (Table 1).

Finally, to determine whether other *ama-1* antisense RNAs would cause the same phenotype, we tested two smaller nonoverlapping RNAs transcribed from shorter cDNA constructs (pJ101 and pJ102; see Fig. 1). Both caused the early arrest phenotype, although not as efficiently as the DB19 RNA (Table 2). Higher concentrations of the smaller

TABLE 1
Phenotypes Resulting from Maternal Injection of *ama-1* Antisense and Control RNAs

Injected RNA	Concentration (ng/μl)	% Early arrest	% Late arrest	% Viable L1s	Embryos scored
DB19 *ama-1*	2	80	2.0	18	1353
Antisense	5	96	1.5	2.9	5057
DB19 *ama-1*	2	6.6	0.7	93	928
Sense	5	20	2.6	77	975
pAS07 *her-1*	50	0.7	0.7	99	3126
Antisense	340	0.3	1.7	98	1872

Note. Wild-type or transgenic *vet-6*::*lacZ* hermaphrodites were injected with the RNAs indicated, and progeny embryos produced after a recovery period of 12–16 hr were scored (see Materials and Methods). Embryos from the two different hermaphrodite lines showed no significant differences in percentages of the various phenotypes and have been pooled in the table. Arrested embryos were categorized as "early arrest": <300 cells and no apparent morphogenesis; "late arrest": >300 cells with visible signs of morphogenesis. Numbers in the "viable L1" column are embryos that hatched to produce viable larvae.

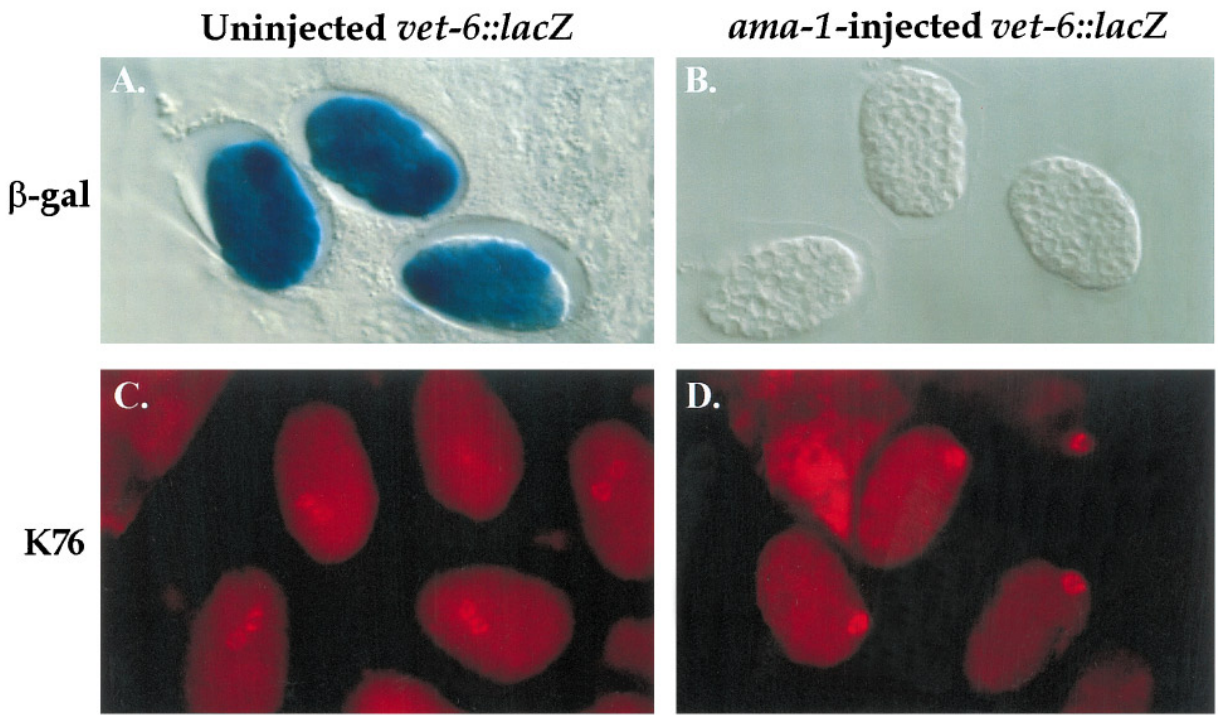


FIG. 2. Expression of an embryonically expressed reporter gene and P granule localization in control and *ama-1* antisense embryos. In all four panels, embryos are at approximately the 100-cell stage. (A, B) Embryos from a transgenic strain carrying an integrated *vet-6::lacZ* reporter gene, viewed with Nomarski optics. Embryos from uninjected hermaphrodites (A) show X-Gal staining in almost all blastomeres. Embryos from hermaphrodites injected with DB19 *ama-1* antisense RNA (Fig. 1) show no detectable staining. (C, D) Embryos from a wild-type strain stained with the anti-P granule monoclonal antibody K76 and viewed with fluorescence optics. Both normal embryos from uninjected hermaphrodites (C) and arrested embryos from *ama-1* antisense-injected hermaphrodites (D) exhibit P granule staining in the expected number of cells, consistent with normal P granule segregation in early cleavages. In the normal embryos, P granule staining is observed in the two germ line cells, Z2 and Z3, whereas in arrested embryos, staining is seen only in their parent cell P_4, which does not divide.

RNAs were required, and their effect was less persistent: injected hermaphrodites began to produce viable embryos again several hours after injection. Therefore, the lower percentages of lethality compared to those observed with DB19 RNA at least partly reflect the shorter period of time during which the smaller RNAs are active.

The inhibitory effect of pJ101 antisense RNA was stronger and persisted longer than that of pJ102 antisense RNA. A third small antisense RNA, transcribed from DB17, with the same 5′ end as pJ102 but including sequence antisense to the entire 3′ UTR and 18 nucleotides of the poly(A) tail (Fig. 1) was essentially inactive in causing early embryonic arrest (Table 2). The DB19 RNA was used in all subsequent experiments.

ama-1 Antisense RNAs Do Not Appear to Inhibit Maternally Controlled Events

Early events following fertilization, such as partitioning of cytoplasmic components to establish anterior–posterior polarity and patterning of the first few cleavages, are known to depend substantially if not entirely on the function of maternally supplied components, as described in the Introduction. Because function of the *ama-1* gene is known to be required during oogenesis as well as in the embryo, the embryonic arrest caused by *ama-1* antisense RNA injection into maternal gonads might result from decreased maternal transcription of genes that encode these components. As a test of this possibility, we analyzed *ama-1* antisense embryos for the occurrence of the maternally controlled events that lead to execution of the first two cleavages and partitioning of the P granules to the germ line cell during each of the first four rounds of cell division (Strome and Wood, 1983). All these events require maternal function of some or all of the *par* genes (Kemphues *et al.*, 1988; Etemad-Moghadam *et al.*, 1995).

To analyze characteristics of the first cleavages, we used the 4D microscope (see Materials and Methods) to record development of normal and *ama-1* antisense embryos beginning at the 2-cell stage. We monitored cleavage plane

TABLE 2
Phenotypes Resulting from Maternal Injection of Smaller *ama-1* Antisense RNAs

Injected RNA	Concentration (ng/μl)	% Early arrest	% Late arrest	% Viable L1s	Embryos scored
pJ101 *ama-1*	5	1.5	3.1	95	1423
Antisense	50	65	25	11	1932
	200	79	18	2.9	1813
pJ102 *ama-1*	50	11	15	74	1935
Antisense	200	20	30	50	1141
DB17 *ama-1*	100	0.06	0.4	99	1582
Antisense	1000	3.7	13	83	1095

Note. Wild-type hermaphrodites were injected with the indicated antisense RNA and progeny embryos produced between 15 and 39 hr postinjection were scored. The effects of pJ101 and pJ102 RNAs were less persistant than that of DB19 RNA. pJ101 injection usually resulted in arrested progeny throughout the scoring period. However, hermaphrodites injected with pJ102 often appeared to recover, producing only viable progeny after 24 to 30 hr postinjection. This behavior accounts only in part for the lower levels of lethality seen with pJ102 than with pJ101.

orientations, size inequality of daughter cells, and timing of cell divisions. In embryos from hermaphrodites defective in function of the *par* genes (Kemphues *et al.*, 1988) or any of several other less well-characterized genes identified by strict maternal-effect mutations (Wood *et al.*, 1980; Schierenberg *et al.*, 1980), all these characteristics are markedly abnormal. However, all nine of the *ama-1* antisense embryos examined were indistinguishable in these characteristics from control embryos produced by uninjected hermaphrodites.

To examine partitioning of P granules, we stained *ama-1* antisense embryos at various early stages with the K76 monoclonal antibody, which recognizes a P-granule epitope (Strome and Wood, 1983). In normal embryos, the P granules are partitioned to the P_1 cell at first cleavage and thereafter to the P_2, P_3, and P_4 germ line daughters in each of the subsequent three cleavages. When the P_4 cell divides after a delay of about 70 min, the P granules are inherited by both the germ line daughter cells, Z2 and Z3 (Strome and Wood, 1993), which can be seen as the only two cells staining with K76 antibody in a 100-cell embryo (Fig. 2C). In embryos from hermaphrodites defective in *par-1, par-2, par-3, par-4,* or *par-6* function, the initial partitioning is aberrant and P granules are generally not detectable by immunofluorescence staining after the first few cleavages (Kemphues *et al.*, 1988; K. Kemphues, personal communication).

When we assayed embryos fixed at various stages from two to >100 cells by immunofluorescence using K76 antibody, we observed staining in 78% of *ama-1* antisense embryos (n = 163) compared to 88% of control embryos (n = 262). In all these embryos, the P granules were normal in appearance and were localized appropriately (data not shown for early stages). In the arrested *ama-1* antisense embryos, the germ line precursor P_4 does not divide (see below). In these embryos, K76 staining was always restricted to a single cell (Fig. 2D).

These results indicate that *ama-1* antisense RNA injection into hermaphrodite gonads does not detectably perturb any of several early maternally controlled events in progeny embryos, suggesting that it acts specifically to inhibit expression of *ama-1* and not other maternally transcribed genes. Therefore, the effects of *ama-1* antisense RNA injection on later events in embryogenesis are likely to be the result of decreased embryonic transcription.

Cellular Defects in ama-1 Antisense Embryos

To determine the nature of the defects leading to early embryonic arrest, we analyzed recordings of nine *ama-1* antisense embryos from the 4-cell stage until after the arrest of cell division. All of the embryos exhibited similar phenotypes. Through the first four cell cycles, the patterns of cleavage were indistinguishable from those of control embryos from uninjected wild-type hermaphrodites, and the relative timing of divisions between the various lineages was normal (Fig. 4). The first clearly apparent defect was aberrant behavior of the endodermal precursors, Ea and Ep, during the fifth cell cycle (26-cell stage). In normal 26-cell embryos, the Ea and Ep cells begin to move from the ventral surface into the blastocoel. At the end of this short migration, they divide once in a dorsal–ventral direction (Figs. 3A and 3B). As cell division and gastrulation continue, the mesodermal (MS, C, and D descendants) and germ line (P_4) precursors follow the E cells into the interior of the embryo (Sulston *et al.*, 1983). In the *ama-1* antisense embryos, Ea and Ep exhibited a shortened cell cycle resulting in premature division prior to that of the AB and C descendants, which normally divide first (Fig. 4; see below). Ea and Ep also oriented their spindles inappropriately, so that they divided in an anterior–posterior direction. The resulting four E cells did not move into the blastocoel (Fig. 3E); they subsequently divided once more prior to cell division arrest but remained on the ventral surface of the embryo (Fig. 3F). The mesodermal and germ line precursors also failed to migrate into the interior of the embryo. Therefore, the earliest defects seen in *ama-1* antisense embryos are premature,

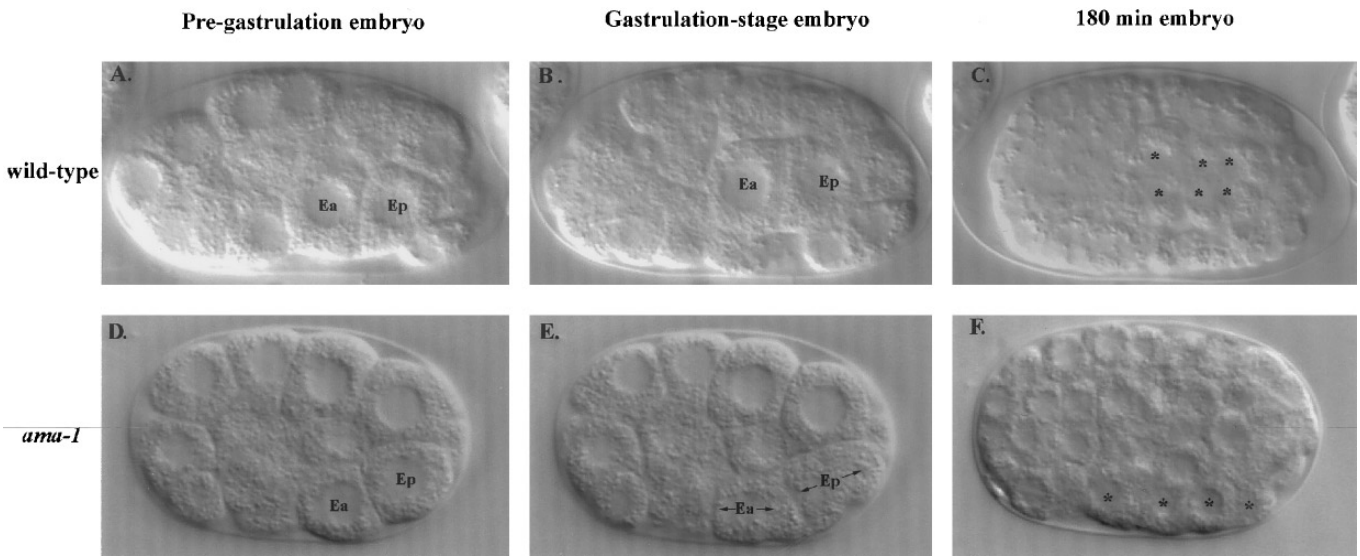


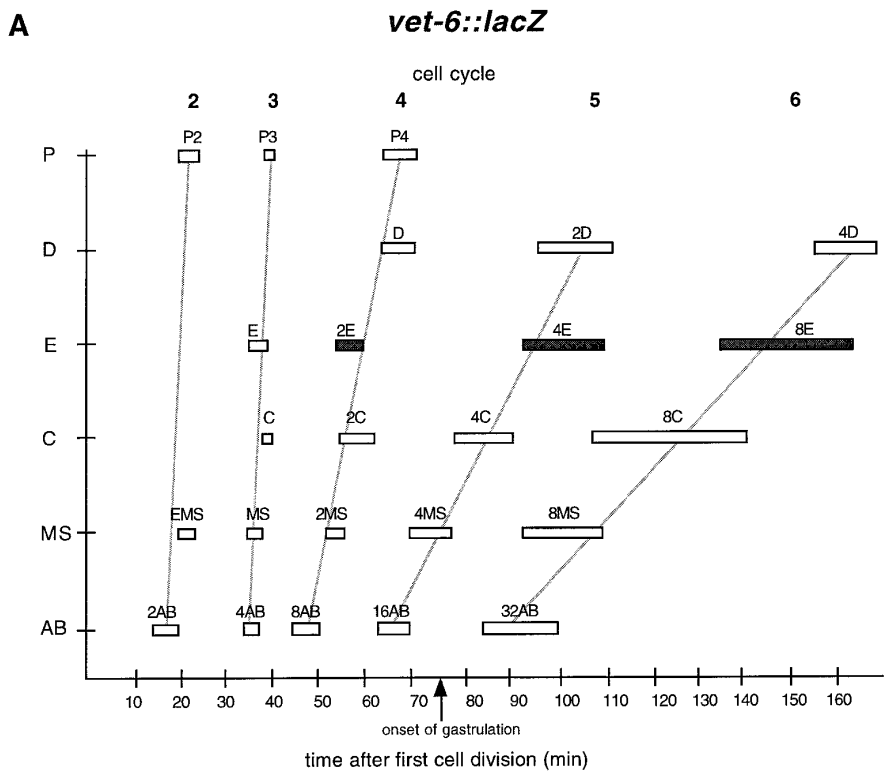
FIG. 3. Development of wild-type and *ama-1* antisense embryos. Nomarski images of three different stages are shown for a wild-type embryo (top panels) and an *ama-1* antisense embryo (bottom panels). All images are anterior-left, dorsal-up. The two embryos are oriented slightly differently, such that the germ line cell, P_4, is out of the plane of focus in the *ama-1* antisense embryo. Endodermal precursors are labeled. Prior to gastrulation onset (A, D), the embryos appear identical. In the wild-type embryo (B), gastrulation begins when the Ea and Ep cells migrate interiorly. In *ama-1* antisense embryos (E), Ea and Ep remain on the ventral surface, and they divide precociously and aberrantly (arrows) in the anterior–posterior direction. By 180 min after first cleavage, the two embryos look significantly different. In the wild type (C), the eight E descendants are lined up in two rows in the interior of the embryo (asterisks), where they will later differentiate into gut. In the *ama-1* antisense embryo (F), which has arrested, the eight E descendants are still on the outer ventral surface of the embryo (asterisks). In both embryos, not all of the eight E descendants are in the plane of focus. Images were taken from video recordings made with the 4D microscope.

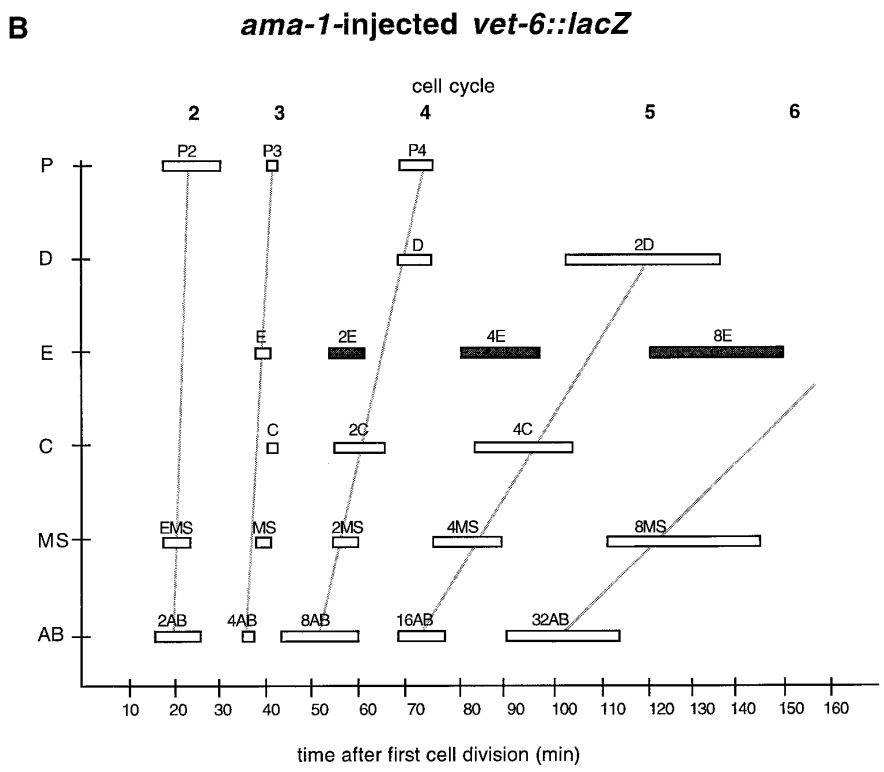
aberrant division of Ea and Ep and failure to initiate gastrulation. These defects are apparent at about 80 min after first cleavage, more than an hour before the general arrest of cell division.

The 9 *ama-1* antisense embryos arrested between the sixth and seventh cell cycles. The blastomere division times for 7 of these embryos are diagrammed in Fig. 4. The 2 other embryos slowed development after the fourth cell cycle, but the order and orientations of cell divisions were the same in all 9 embryos. Although the E blastomeres divided early, they completed the sixth cell cycle, as did the AB and MS lineages. However, in most of the embryos, the C and D descendants did not divide during the sixth cell cycle. The C descendants divided to produce 8 cells in 4 of the 9 embryos, and the D blastomeres divided from 2 to 4 cells in only 1 of the embryos within 180 min after first cleavage. Only the AB blastomeres completed the seventh cell cycle (to produce 64 AB cells) in all 9 embryos. Occasionally, individual blastomeres would divide once more with unpredictable timing. The mean number of cells produced in these 9 embryos after 180 min was 91. We stained the nuclei of 22 other arrested *ama-1* antisense embryos with DAPI and determined that these embryos contained a range of 62 to 106 cells, again with a mean of 91 cells. For comparison, untreated embryos at this time consist of about 190 cells.

E-cell descendants in wild-type embryos express rhabditin granules (Babu, 1974; Laufer *et al.*, 1980), detectable as refractile or autofluorescent particles in the gut primordium

FIG. 4. Cell division times during the first six cell cycles for wild-type and *ama-1* antisense embryos. The development of self-progeny embryos from transgenic *vet-6::lac Z* hermaphrodites, either uninjected (A) or injected with DB19 *ama-1* antisense RNA (B), was recorded at about 25°C (see Materials and Methods). The six founder cell lineages are indicated on the vertical axis. Horizontal bars indicate the range of division times in each lineage at each cell cycle; dotted lines connect cell divisions that belong to the same cell cycle. Times in (A) are averaged from recordings of two embryos; times in (B) from recordings of seven embryos. The first cell cycle is not shown because most recordings were initiated after first cleavage had occurred. Division times for the C and D lineages during the sixth cell cycle are omitted in (B) because these divisions did not always occur in *ama-1* antisense embryos (see text). Temperature control in these experiments was not precise enough to determine whether the slightly slower (about 6%) average division rate in (B) was the result of antisense injection or of slightly lower temperatures than in (A).





after the 8-E-cell stage (about 170 min after first cleavage). Appearance of these granules, which is known to require embryonic transcription (Edgar and McGhee, 1988; Edgar *et al.*, 1994), was never observed in arrested *ama-1* antisense embryos even after 300 min.

DISCUSSION

Inhibition of Embryonic Transcription by ama-1 Antisense RNA

Our results indicate that maternally injected *ama-1* antisense RNA can act effectively and specifically to inhibit transcription by RNA polymerase II in progeny embryos of *C. elegans*. This technique may provide a generally useful method for blocking embryonic transcription in other organisms as well.

In our experiments, following injection of the most active antisense RNA, 96% of progeny embryos failed to express detectable amounts of a *lacZ* reporter driven by a strong early embryo promoter, and up to 96% arrested their development at a stage similar to that of the arrest stage observed for α-amanitin-treated embryos (Edgar *et al.*, 1994).

These abnormalities are not likely to be caused by maternal effects of inhibiting transcription during oogenesis, because the progeny embryos from injected hermaphrodites exhibited no defects in the early maternally controlled processes of partitioning P granules and patterning the first cleavages to establish anterior–posterior polarity. It appears, therefore, that the principal effect of maternal *ama-1* antisense RNA injection is to prevent transcription in the embryo, presumably by blocking translation of maternally provided and perhaps also embryonically produced mRNA for the RNA polymerase II large subunit. The success of this method suggests that RNA polymerase II protein itself, which must be abundantly present in the ovary for production of mRNAs during oogenesis, is largely excluded from progeny embryos.

That a small amount of transcription may be occurring in embryos produced following maternal antisense injection is suggested by their stage of arrest with <100 cells. Edgar *et al.* (1994) showed that devitellinized embryos treated with α-amanitin at the 8-cell stage or later in cleavage also arrested with about 100 cells, whereas those treated at the 2-cell stage arrested with a significantly greater number of about 140 cells. These results, and similar findings with *Drosophila* embryos (O'Farrell *et al.*, 1989), were interpreted to suggest that very early embryonically transcribed gene products may be required for the normal elimination of maternal gene products involved in maintaining the cell cycle during cleavage stage. The arrest at <100 cells that we observe following maternal antisense injection suggests that some early embryonic transcription may be occurring, but if so, it is insufficient to allow detectable expression of the *vet-6::lacZ* reporter or the genes required for appearance of rhabditin granules. We conclude that this treatment is effective in blocking most embryonic transcription.

The mechanism by which antisense RNAs inhibit gene expression in *C. elegans* has been investigated by Fire *et al.* (1991). This work demonstrated that expression vectors producing antisense *unc-54* (myosin B) or *unc-22* (twitchin) RNAs in body wall muscle caused a decrease in the synthesis of the corresponding proteins, without decreasing the levels of the *unc-54* and *unc-22* sense RNAs or interfering with the splicing of these transcripts. These results suggested that the antisense RNAs inhibited translation of the complementary sense RNAs by sterically hindering either their transport to the cytoplasm, loading onto ribosomes, or progression of the translation process. Similar mechanisms of antisense inhibition have been described in other biological systems (reviewed in Takayama and Inouye, 1990; Wagner and Simons, 1994).

In our experiments, the most effective antisense RNA, DB19, included sequence antisense to the 5′ end and most of the *ama-1* transcript, but not to the 3′ end (Fig. 1). Two smaller antisense RNAs were less effective, and the duration of their effect was shorter. However, at higher concentrations they were capable of generating the same phenotype as the DB19 antisense RNA, consistent with the mechanisms of inhibition discussed above. We have not attempted to determine whether their lower effectiveness could be due to decreased stability or simply to lower activity as translational inhibitors. However, it may be significant that inclusion of antisense to the 3′ UTR plus 18 nucleotides of the poly(A) tail (DB17) essentially eliminated its inhibitory effect as assayed by arrest phenotype, perhaps because this RNA hybridizes nonspecifically with other mRNAs in the germ line.

With regard to our finding that sense *ama-1* mRNA also shows some inhibitory activity, similar results have been obtained with other *C. elegans* genes. For example, constructs expressing *unc-22* (twitchin) sense RNAs have been reported to cause loss-of-function phenotypes (Fire *et al.*, 1991). This phenomenon is also commonly observed in plant systems, where it has been called cosuppression. While several different explanations for cosuppression have been proposed, the molecular mechanisms are not fully understood (Mol *et al.*, 1991; Hart *et al.*, 1992; Brusslan *et al.*, 1993). In our experiments, the DB19 sense RNA did include the translation initiation codon and, therefore, might have been translated to produce a truncated protein with inhibitory effects. We have not attempted to distinguish this possibility from other possible mechanisms of cosuppression in our experiments.

Early Endodermal Cell Decisions Require Embryonic Transcription

Several maternally expressed genes are known to participate in specification of the E founder cell, which arises at the 8-cell stage and subsequently gives rise to the entire gut and no other tissue. The *par* gene products are required

for patterning of the first two asymmetric cleavages in the embryo, which position the EMS blastomere (mother of the E and MS founder cells) posteriorly and ventrally in the embryo (Kemphues *et al.*, 1988). The *skn-1* gene product, a putative transcription factor similar to the bZIP family, is required to specify the EMS cell fate (Bowerman *et al.*, 1992). Function of the *mex-1* gene is required to localize SKN-1 protein to the posterior blastomeres EMS and P_2, and function of *pie-1* restricts its activity to EMS (Bowerman *et al.*, 1993). At the 4-cell stage, in a process for which the genetic control is still unknown, contact between EMS and the neighboring P_2 cell is required to orient the EMS spindle and induce segregation or activation of gut determinants in the E daughter cell (Goldstein, 1992, 1995).

Once founded, the E lineage exhibits three characteristic behaviors: longer cell cycles than those of the other somatic lineages (see Fig. 4), movement of the E daughters Ea and Ep into the blastocoel at the 26-cell stage to initiate gastrulation, and subsequent Ea and Ep division in a dorsal–ventral direction to initiate formation of the gut primordium. Our results indicate that one or more embryonically expressed genes are required to specify or maintain these characteristics of the E lineage. In *ama-1* antisense embryos, the cell cycles of Ea and Ep are shortened relative to those of the other somatic lineages. Ea and Ep fail to move into the interior and instead divide aberrantly on the ventral surface in an anterior–posterior direction. The responsible embryonically expressed genes are likely to be controlled by maternally expressed genes specifying the E lineage.

To what extent are the three E lineage behaviors listed above dependent on initial specification of the E founder cell? A convenient E lineage differentiation marker is the gut-specific rhabditin granules described above. In maternal-effect-mutant embryos that do not specify an E founder cell as judged by failure to express this gut marker, such as those with *skn-1* or *gut* gene defects, the misspecified E cells divide early and do not move inward on schedule, and gastrulation is aberrant and incomplete (reviewed in Bucher and Seydoux, 1994). Similar phenotypes are also observed as the result of several other maternal-effect mutations, which affect the E lineage cell cycle and gastrulation but do not prevent expression of the E lineage differentiation marker (Schierenberg *et al.*, 1980; Cassada *et al.*, 1981; Denich *et al.*, 1984; reviewed by Bucher and Seydoux, 1994). In particular, *emb-16*-defective embryos have a gastrulation phenotype very similar to that of *ama-1* antisense embryos. [Bucher and Seydoux questioned the *emb-16* phenotype; however, we have obtained the strain used by Denich *et al.* (1984) and confirmed these authors' description of the gastrulation defect.] The only currently known non-maternal-effect mutations affecting gastrulation are those in the *zen-1* gene, which cause early Ea and Ep division, delayed gastrulation onset, and incomplete gastrulation. Consistent with our findings, these defects probably result from a general failure in processing of embryonic transcripts due to lack of the SL1 splice leader sequence in *zen-1* mutant embryos (Ferguson *et al.*, 1996).

Junkersdorf and Schierenberg (1992) have suggested that ability to move inward at the 26-cell stage is a cell-autonomous property of Ea and Ep, since ablation of the neighboring cells in wild-type embryos does not block movement of Ea and Ep to the interior. Therefore, each of the three defects seen in *ama-1* antisense embryos, in cell cycle regulation, cell movement, and spindle orientation, could be caused by absence of one or more gene products in the E lineage. Other studies have shown that these phenotypes are at least partially separable. When the impermeable vitelline membrane that surrounds the embryo is disrupted at the 8-cell stage, Ea and Ep subsequently fail to move toward the interior, although they do not divide until the normal time (Schierenberg and Junkersdorf, 1992). No gastrulation is observed in these embryos, and disruption of the membrane at later times arrests gastrulation, suggesting that membrane integrity is required for the initiation and progression of normal ingression. Conversely, in some of the maternal-effect mutants mentioned above as well as the *zen-1* mutant, E cells do move belatedly to the interior even though the Ea and Ep divisions occur prematurely and in an anterior–posterior direction at the cell surface.

A plausible model consistent with all the currently available evidence is that maternal-effect genes, including those necessary for specification of the E founder cell, act to regulate embryonic expression of one or more E lineage genes whose function is required for lengthening of the cell cycle and initiation of gastrulation movements. Genetic screens to identify such genes are in progress.

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